Chapter 3

Endothelial–cardiomyocyte crosstalk enhances pharmacological cardioprotection

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ABSTRACT

Endothelial cells (EC) serve a paracrine function to enhance signaling in cardiomyocytes (CM), and conversely, CM secrete factors that impact EC function. Understanding how EC interact with CM may be critically important in the context of ischemia–reperfusion injury, where EC might promote CM survival. We used isoflurane as a pharmacological stimulus to enhance EC protection of CM against hypoxia and reoxygenation injury. Triggering of intracellular signal transduction pathways culminating in the enhanced production of nitric oxide (NO) appears to be a central component of pharmacologically induced cardioprotection. Although the endothelium is well recognized as a regulator for vascular tone, little attention has been given to its potential importance in mediating cardioprotection. In the current investigation, EC–CM in co-culture were used to test the hypothesis that EC contribute to isoflurane-enhanced protection of CM against hypoxia and reoxygenation injury and that this protection depends on hypoxia-inducible factor (HIF1α) and NO. CM were protected against cell injury [lactate dehydrogenase (LDH) release] to a greater extent in the presence vs. absence of isoflurane-stimulated EC (1.7±0.2 vs. 4.58±0.8 fold change LDH release), and this protection was NO-dependent. Isoflurane enhanced release of NO in EC (1103±58 vs. 702±92 pmol/mg protein) and EC–CM in co-culture sustained NO release during reoxygenation. In contrast, lentiviral mediated HIF1α knockdown in EC decreased basal and isoflurane stimulated NO release in an eNOS dependent manner (517±32 vs. 493±38 pmol/mg protein) and prevented the sustained increase in NO during reoxygenation when co-cultured. Opening of mitochondrial permeability transition pore (mPTP), an index of mitochondrial integrity, was delayed in the presence vs. absence of EC (141± 2 vs. 128±2.5 arbitrary mPTP opening time). Isoflurane stimulated an increase in HIF1α in EC but not in CM under normal oxygen tension (3.5±0.1 vs. 0.79±0.15 fold change density) and this action was blocked by pretreatment with the Mitogen-activated Protein/Extracellular Signal-regulated Kinase inhibitor U0126. Expression and nuclear translocation of HIF1α were confirmed by Western blot and immunofluorescence. Taken together, these data support the concept that EC are stimulated by isoflurane to produce important cardioprotective factors that may contribute to protection of myocardium during ischemia and reperfusion injury.

1. Introduction

Endothelial cell–cardiomyocyte (EC–CM) interactions play a key role in regulating cardiac function by modulating vascular tone and by stimulating proliferation of neighboring cells [1]. CM are surrounded by a capillary network which is critical for maintaining a constant supply of oxygen and nutrients [2]. However, this arrangement of CM and EC in the heart also allows for cell-to-cell signaling between CM and EC which may be of significance during cellular stresses (e.g. ischemia and reperfusion). Importantly, the release of paracrine and autocrine factors is likely to contribute to endogenous and pharmacological cardioprotective pathways [3]. Volatile anesthetic agents such as isoflurane, produce remarkable protective effects to decrease the extent of myocardial infarction after coronary artery occlusion and reperfusion when administered either before (anesthetic preconditioning; APC) [4], or after (anesthetic postconditioning) [5] index ischemia. Considerable progress has been made in uncovering mechanisms responsible for the protective actions of pharmacological pre- and post-conditioning agents including activation of pro-survival signaling pathways [6,7] and preservation of mitochondrial function [8–10]. However, the clinical benefit of such strategies in humans is not clear and may be dependent on age and/or co-existing pathology [11–13]. For example, pre- and post-conditioning appears to be less effective in patients who are elderly [14], or in those patients with diabetes [15]. Endothelial dysfunction that accompanies these conditions could represent a final common denominator that predicts impaired cardioprotective signaling [16]. Nitric oxide (NO) is a likely- paracrine factor that relays signals between EC and CM during cardioprotection. Evidence clearly indicates that endothelial nitric oxide synthase (eNOS) derived NO is a critical component of APC-induced signal transduction [17], however until now the distinct contribution of EC versus CM to NO signaling has not been evaluated. Isoflurane has been shown to activate eNOS, as indicated by phosphorylation of serine 1177, resulting in increased NO production [18]. The non-selective nitric oxide synthase (NOS)-inhibitor N-nitro-L-arginine methyl ester (L-NAME) blocked early APC [17] and isoflurane failed to protect against myocardial infarction or mitochondrial transition pore (mPTP) opening in eNOS–/– mice [19]. Additionally, the trigger and mediator phases of delayed APC were blocked by L-NAME, whereas, specific inhibitors of inducible or neuronal NOS had no effect [20]. The mechanisms responsible for isoflurane-induced NO production in EC are incompletely defined. One possible

candidate protein for activating preconditioning-related pathways is hypoxia-inducible factor (HIF1α) [21,22].

We tested the hypothesis that pharmacological preconditioning with isoflurane is differentially mediated by HIF1α in EC and CM and that EC–CM crosstalk promotes cardioprotection.

2. Material and methods

2.1. *Cell culture*

Human coronary artery EC isolated from healthy donor coronary arteries (Cell Applications, San Diego, CA, USA), were cultured at 37 °C in Meso-Endo cell growth medium (Cell Applications), and used for experiments between the 4th and 6th passages when approximately 70–80% confluent. In some experiments, EC were treated with U0126, (10 μM) or PD98059 (10 μM) (EMB Biosciences, Gibbstown, NJ, USA), two chemically distinct inhibitors of MEK, an upstream kinase that phosphorylates extracellular signal-regulated kinase (ERK1/2) for 60 min before isoflurane, hypoxia or dimethyloxaloylglycine (DMOG) treatment. Neonatal rat CM were isolated from one-day-old Wistar rat hearts by repeated enzyme digestion (0.15 mg/ml collagenase II and 0.52 mg/ml pancreatin, Sigma-Aldrich, St Louis, MO, USA) and centrifugation as described [23]. Cells were used for experiments 3– 7 days after isolation when demonstrating rhythmic contractions

2.2. *Co-culture*

EC and CM were combined at a ratio of 1:12, respectively, 12 h before the experiment with the same number of CM used in all experimental groups (Fig. 1). This ratio was chosen after performing pilot experiments to determine the appropriate ratio whereby EC by themselves had no effect to enhance protection of CM against hypoxia and reoxygenation injury. Isoflurane (1.6%) was administered for 60 min via a vaporizer using air as a carrier at 2 L/min as previously described [17]. The vapor phase anesthetic concentration was continuously monitored by a gas analyzer (POET IQ; Criticare System, Waukesha, WI, USA), and the liquid phase concentration by gas chromatography. Equilibration of isoflurane in the media was 0.5 mM under these conditions. Because gas flow can induce shear-stress-dependent NO release, the control group was exposed to air alone at

the same flow rate. After a 15 min memory period, cells were exposed to 120 min hypoxia (0.1% O_2, Biospherix hypoxia chamber, Lacona, NY, USA) in glucose-free medium and afterwards subjected to 120 min of reoxygenation. L-NAME (1 mM, Sigma-Aldrich) was used to inhibit NOS. Cell damage was assessed by quantification of lactate dehydrogenase (LDH) release in the medium using a commercially available kit (Genzyme Diagnostics, Cambridge, MA, USA) and trypan blue (Sigma) exclusion. Cells that excluded trypan blue dye were considered viable and expressed as a percentage of total cells. Experiments during method establishment indicated that EC do not contribute significantly to LDH release in our experimental protocol.

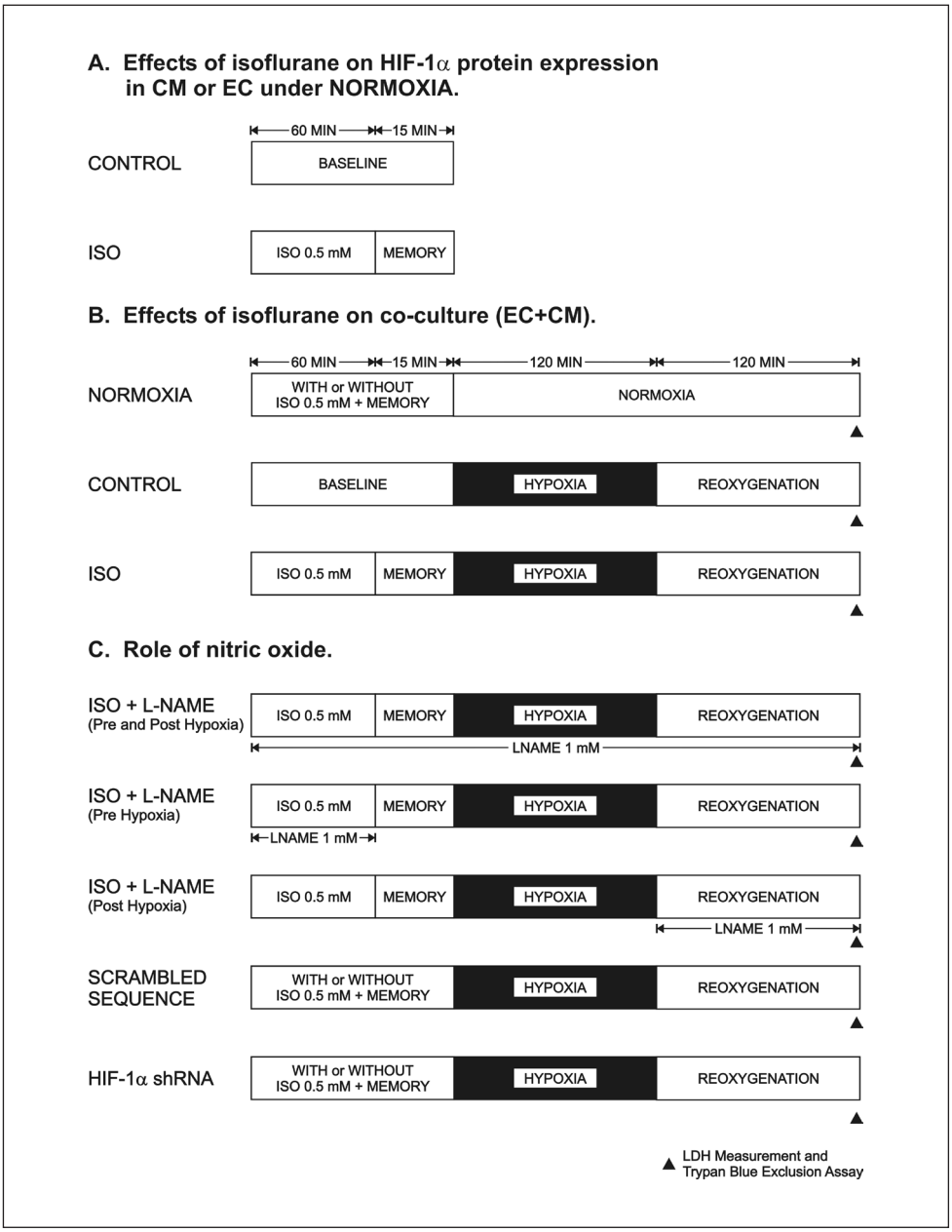

Fig. 1. (A) Schematic diagram of experimental protocols. Effect of hypoxia–reoxygenation, in the presence or absence of isoflurane (ISO), on cell survival measured by lactate dehydrogenase (LDH) activity in co-culture (CM+EC). (B) Effect of the addition of NG-nitro-L-arginine methyl ester (L-NAME) at different time points during the experimental protocol.

2.3. *Immunoblotting*

Total soluble protein from cell lysates was prepared as previously described [16]. Subcellular fractionation was performed using a nuclear and cytoplasmatic extraction kit (Thermo Fisher-Scientific, Rockford, Illinois, USA). Fifty microgram of protein was resolved on a 7.5% SDS-polyacrylamide gel, proteins transferred to polyvinylidene fluoride membranes, and the membranes blocked in tris-buffered saline containing 5% milk. The membranes were incubated with primary antibodies against HIF1α (GeneTex, GTX16535, Irvine, CA, USA) overnight at 4 °C, washed and then incubated with the appropriate secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence followed by densitometric analysis using image acquisition and analysis software (Image J, NIH). β-Actin (Abcam, ab6276, Cambridge, MA, USA) and TATA binding protein (Abcam, ab818) were used as cytoplasmic and nuclear markers, respectively for normalizing HIF1α expression. In addition CD-31 (Abcam, ab28364) for normalizing EC protein expression and alpha-myosin heavy chain (MHC; Santa Cruz, Santa Cruz, CA, USA) for normalizing CM protein expression were used where appropriate.

2.4. *Immunofluorescence*

Cells were cultured on gelatin-coated slides as previously described [17] to visualize expression and translocation of HIF1α. Cells were fixed in 1% paraformaldehyde, permeabilized in 0.5% TritonX-100 and incubated with appropriate antibodies, as described above, in phosphate-buffered saline followed by incubation with corresponding biotinylated secondary antibody (Santa Cruz) for 30 min at 37 °C. Cell nuclei were subsequently stained using TO-PRO-3 (Invitrogen, Carlsbad, CA, USA) for 5 min at room temperature. Images were acquired by confocal microscopy.

2.5. *Confocal microscopy*

Cells were visualized using an inverted laser scanning confocal microscope (Nikon Eclipse TE 200-U microscope with EZ C1 laser scanning software) with a ×40/1.3 oil-immersion objective. Fluorescent probes were excited at 488 nm with an argon laser and at 543 nm with a green helium–neon laser, and a set of filters (ND4 and ND8) was used to minimize dye

bleaching. Data were analyzed using MetaMorph 6.1 software (Molecular Devices, Sunnyvale, CA, USA).

2.6. *Ozone chemiluminescence*

Nitrite concentration (an index of NO) was measured in the cell culture medium using a Sievers NO gas analyzer (Model 280, GE Analytical Instruments, Boulder, CO, USA) as previously described [24]. Briefly, cell culture media (1 ml) from EC, CM and co-culture were collected at three time points (after 60 min of isoflurane exposure or 60 min of baseline for control dishes; after 120 min of hypoxia; and after 120 min of reperfusion) and immediately frozen in liquid nitrogen. Nitrite concentration was calculated after subtraction of background levels and normalized to total cell protein from cell lysates prepared as described for Western blotting.

2.7. *Mitochondrial membrane potential*

Cells were incubated with the mitochondrial membrane potential ($\Delta\Psi m$) fluorescent probe tetramethylrhodamine (TMRE; 100 nM) for 30 min as previously described [10]. Briefly, TMRE was included in the superfusing solution throughout the experiments. TMRE fluorescence intensity was recorded at 590 nm (excitation by green helium–neon laser), and the changes in $\Delta\Psi m$ were monitored by calculating relative TMRE fluorescence. Cell cultures were subjected to laser-induced oxidative stress until mPTP opening occurred [25]. In some experiments, after incubation with TMRE, CM were loaded with calcein-AM (1.0 μM; Invitrogen) and cobalt chloride (2 mM) in order to verify the opening of mPTP independently from changes of $\Delta\Psi m$. Opening of mPTP was visualized as a collapse of $\Delta\Psi m$ and release of the fluorescent dye calcein from mitochondria. Measurements in the presence of cyclosporine A (1 μM) were used to determine specificity of mPTP-opening.

2.8. *Gene silencing*

Lentiviral particles containing HIF1α (Thermo Fisher-Scientific) or its nucleotide-substituted control were packaged and produced as previously described [26]. Briefly, 10 μg HIF1α or control shRNA plasmid, 6.5 μg porcine cytomegalovirus (p-CMV) R8.74 as packing construct, and 3.5

μg vesicular stomatitis virus glycoprotein-G (VSV-G) carrying the sequence for the envelope, were co-transfected into 293T cells using the calcium-phosphate co-precipitation method. Medium was replaced after 12–14 h. After 36–48 h, the replication-defective lentiviral vectors were harvested, cleared by low-speed centrifugation, and filtered through 0.45 μm cellulose acetate filters. The viral titer was determined by FACS analysis of GFP-positive 293T cells. Pilot experiments using a GFP expressing lentiviral vector were performed to determine the optimal multiplicity of infection (MOI) and were found to be 10 (95–100% GFP-positive EC). EC were infected by three consecutive additions of the viral particles 24 h apart at a dose of 4×10^5 transducing units per well in a 24-well plate.

2.9. *Statistics*

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by the Student–Newman–Keuls test. The assumption of normality was evaluated by examining the residual diagnostic plots; no substantial deviations were found. Sample sizes for the experimental groups were 6–8 with 3 replicates per group. Changes were considered statistically significant when $P < 0.05$. All data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. *Isoflurane modulates HIF1α regulation in EC but not in CM*

Isoflurane increased HIF1α expression in EC in a time dependent fashion (Fig. 2A) and expression was maximal after 60 min of isoflurane treatment. Therefore, translocation of HIF1α between cytosolic and nuclear compartments was assessed 60 min after isoflurane for the remaining experiments. Increases in HIF1α after isoflurane treatment were observed in the cytosolic and nuclear fraction as detected by immunofluorescence (Fig. 3A) and Western blotting (Fig. 3B). This action was blocked by two different MEK inhibitors, UO126 (Fig. 3) and PD98059 (data not shown). In contrast, HIF1α expression was increased in EC subjected to hypoxia or treated with the prolyl hydroxylase inhibitor DMOG and this increase was not inhibited by pretreatment with UO126 (Supplemental data Fig. 1).

These results indicate that the regulation of HIF1α expression in EC by isoflurane is dependent on activation of the pro-survival kinase ERK1/2. There were no differences in the expression of HIF1α in CM treated with isoflurane as compared to untreated cells (Fig. 2B).

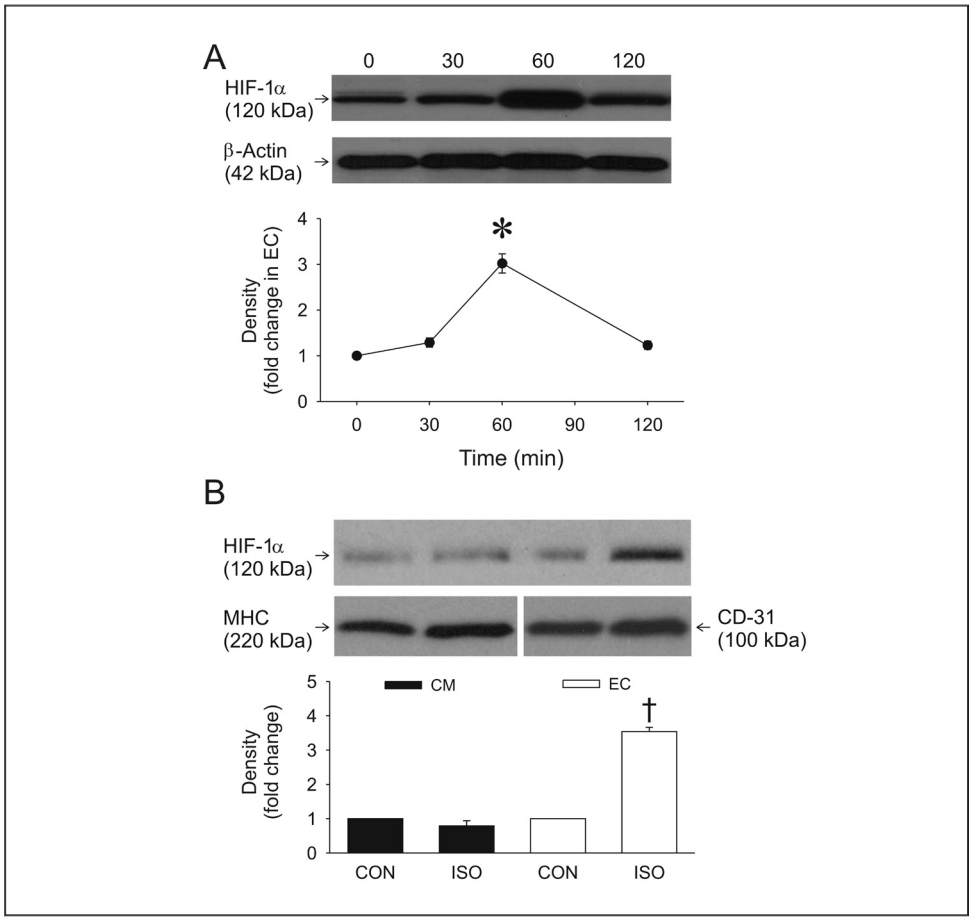

Fig. 2. (A) Representative Western blot illustrating temporal expression of hypoxia-inducible factor (HIF1α) in isoflurane treated endothelial cells (EC).
HIF1α protein amount is expressed as fold change of control.

(B) Expression of HIF1α in cardiomyocytes (CM) and EC treated with isoflurane (ISO). HIF1α protein amount is expressed as fold change to respective untreated control (CON).
*Data are mean ± SEM (n=6/group); *P < 0.05 vs. baseline, 30 or 120 min of ISO exposure, †P < 0.05 vs. CON EC.*

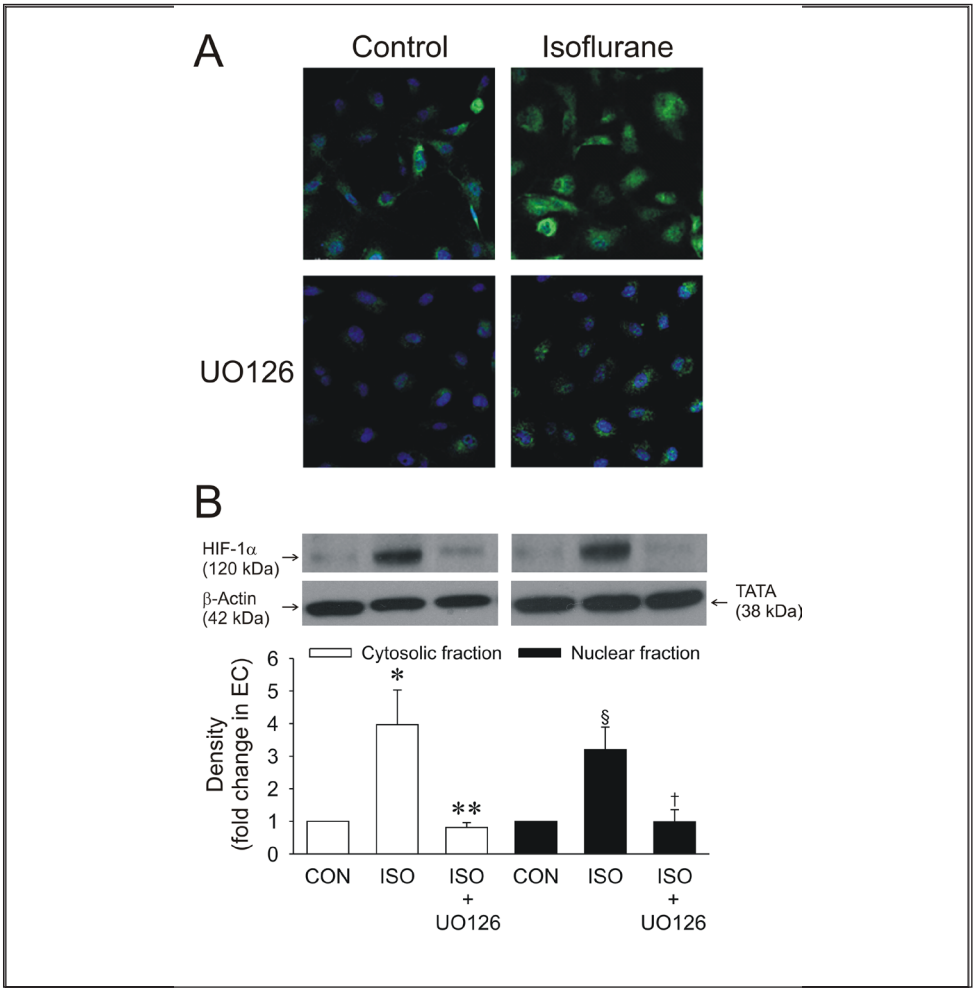

Fig. 3. (A) Immunofluorescence depicting expression and subcellular localization of hypoxia-inducible factor (HIF1α) in endothelial cells (EC) in the presence and absence of isoflurane and pretreated with the Mitogen-activated Protein/Extracellular Signal-regulated Kinase inhibitor UO126 (green = HIF1α, blue = DAPI nuclear staining). (B) Representative Western blot studies showing cytosolic and nuclear HIF1α protein expression in EC with and without isoflurane (ISO) and in the presence of UO126 (ISO+ UO126).

*HIF1α protein amount is expressed as fold change of respective untreated control (CON). Data are mean ± SEM (n=6/group); *P < 0.05 vs. cytosolic CON, **P < 0.05 vs. cytosolic ISO, §P< 0.05 vs. nuclear CON, †P< 0.05 vs. nuclear ISO.*

3.2. *Isoflurane-mediated protection of CM is enhanced by co-culture with EC*

EC were resistant to hypoxia and reoxygenation (H/R) injury and lactate dehydrogenase (LDH) release from EC was unchanged by H/R as compared to normoxic conditions (Fig. 4A). These findings were similar to those previously reported [27] and were verified by trypan blue exclusion (Supplemental data Fig. 2). Co-culture of EC with CM (1:12) had no effect on LDH release after H/R compared to CM alone. In contrast, isoflurane significantly reduced LDH release from CM in co-culture, whereas isoflurane had no effect on cell injury in the absence of EC. These findings suggest that EC contribute to protection of CM against H/R and that the contribution of EC to protection is modulated by isoflurane. The dependence of this favorable interaction between EC and CM on NO was examined using the non-selective NOS inhibitor, L-NAME, which was incubated either before hypoxia, upon reoxygenation, or throughout the entire experiment. L-NAME abolished reduction of LDH release in co-culture when present throughout the experimental protocol, or during reperfusion, but not when present prior to hypoxia alone (Fig. 4B). These results indirectly indicate that NO produced during reoxygenation is critically important in mediating pharmacologically-enhanced EC protection of CM.

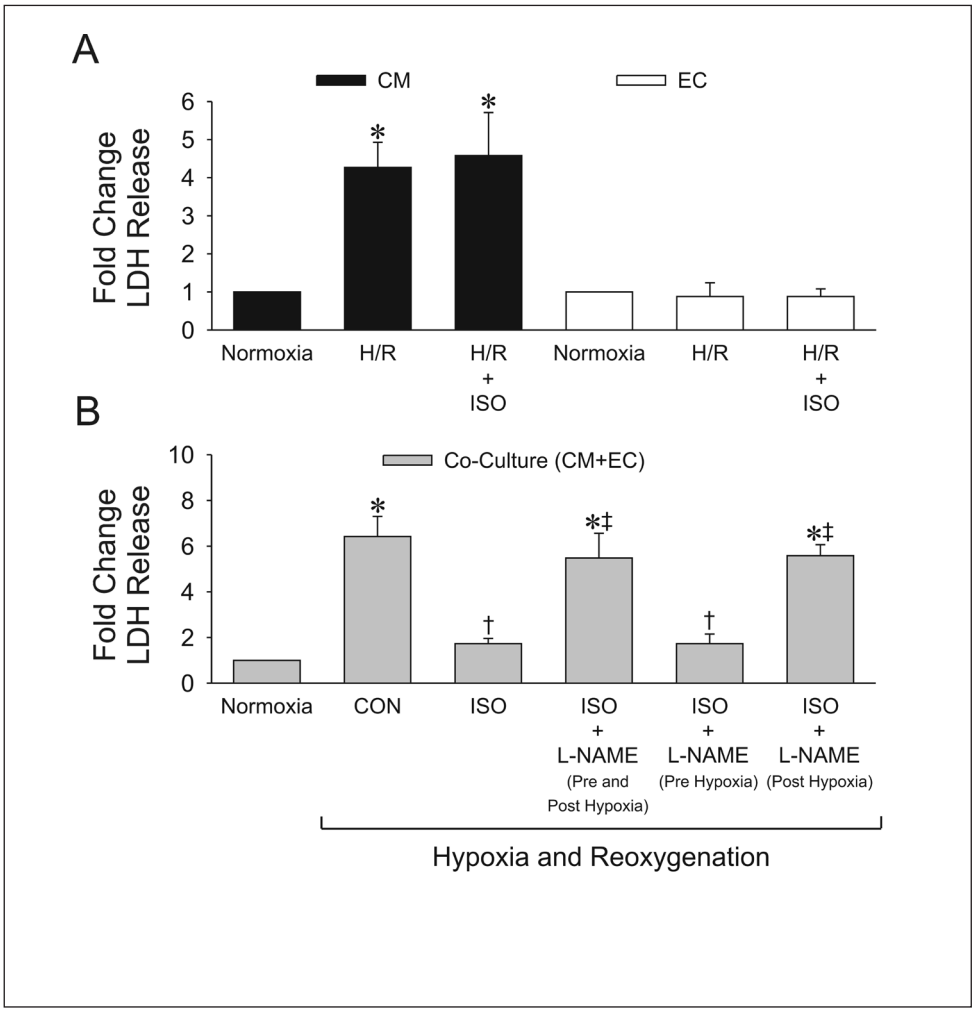

Fig. 4. (A) Histograms depicting lactate dehydrogenase (LDH) activity in cardiomyocytes (CM) and endothelial cells (EC) undergoing hypoxia–reoxygenation (H/R) or normoxia in the presence (H/R + ISO) or absence (H/R) of isoflurane. Panel (B) summarizes the effects of H/R in the presence (ISO) or absence (CON) of isoflurane on co-culture (CM+EC) with or without the addition of NG-nitro-L-arginine methyl ester (L-NAME).

*LDH activity is expressed as fold change over the respective normoxic-group. Data are mean ± SEM (n=8/group); *P < 0.05 vs. respective normoxia, †P < 0.05 vs. CON, ‡P < 0.05 vs. ISO.*

3.3. *NO production is enhanced in EC–CM co-culture*

During normoxic conditions, isoflurane significantly increased NO production in treated as compared with untreated EC, a finding that is consistent with our previous report [17]. This production of NO was inhibited by the non-selective NOS inhibitor L-NAME, suggesting that the source of NO is likely NOS. In contrast, isoflurane did not alter NO production during normoxia in CM alone or in co-culture (Table 1). The production of NO, measured as nitrite, is predominantly extracellular NO as measurement of intracellular nitrite was undetectable in EC and represented approximately 10% of total nitrite produced by CM. NO production was significantly decreased during hypoxia in EC, CM and in co-culture. Interestingly, isoflurane preserved NO-production to a greater extent during hypoxia in EC and CM in co-culture compared to the absence of anesthetic or in EC alone (Fig. 5A). During reoxygenation, co-culture of EC and CM sustained NO production at baseline values as compared to EC alone (Fig. 5B), but this action was not further augmented by isoflurane.

Table 1 Nitric oxide-production (pmol/mg protein) by endothelial cells, cardio-myocytes and in co-culture.

	Baseline	Hypoxia	Reoxygenation
EC			
CON	702±92	158±53[a]	338±61[a]
ISO	1103±58[b]	146±50[a]	531±22[a]
EC L-NAME			
CON	532±151	135±21[a]	365±114[a]
ISO	483±77	154±31[a]	236±38[a]
EC SCR			
CON	670±9	204±28[a]	335±15[a]
ISO	1071±42[b]	219±25[a]	614±40[a]
EC shRNA			
CON	517±32	209±13[a]	248±40[a]
ISO	493±38	192±20[a]	205±33[a]
CM			
CON	191±5	110±18[a]	167±26
ISO	197±7	104±12[a]	160±17
CC (EC+CM)			
CON	182±4	54±6[a]	180±23
ISO	222±15	101±5[ab]	214±24
CC (EC+CM) L-NAME			
CON	201±6	24±12[a]	80±12[a]
ISO	176±12	30±16[a]	86±35[a]
CC (EC+CM) SCR			
CON	210±11	89±6[a]	190±5
ISO	250±57	150±13[ab]	210±16
CC (EC+CM) shRNA			
CON	169±8	15±5[a]	103±7[a]
ISO	150±13	21±14[a]	112±9[a]

EC = endothelial cells, EC (SCR) = EC infected with lentivirus expressing scrambled shRNA, EC (shRNA) = EC infected with lentivirus expressing shRNA for hypoxia-inducible factor, CM = cardiomyocytes, CC = co-culture (CM and EC cultured together), CON = control, ISO = isoflurane; data are mean ± SEM (n=6/group).

a Significantly ($P < 0.05$) different from baseline.

b Significantly ($P < 0.05$) different from respective control.

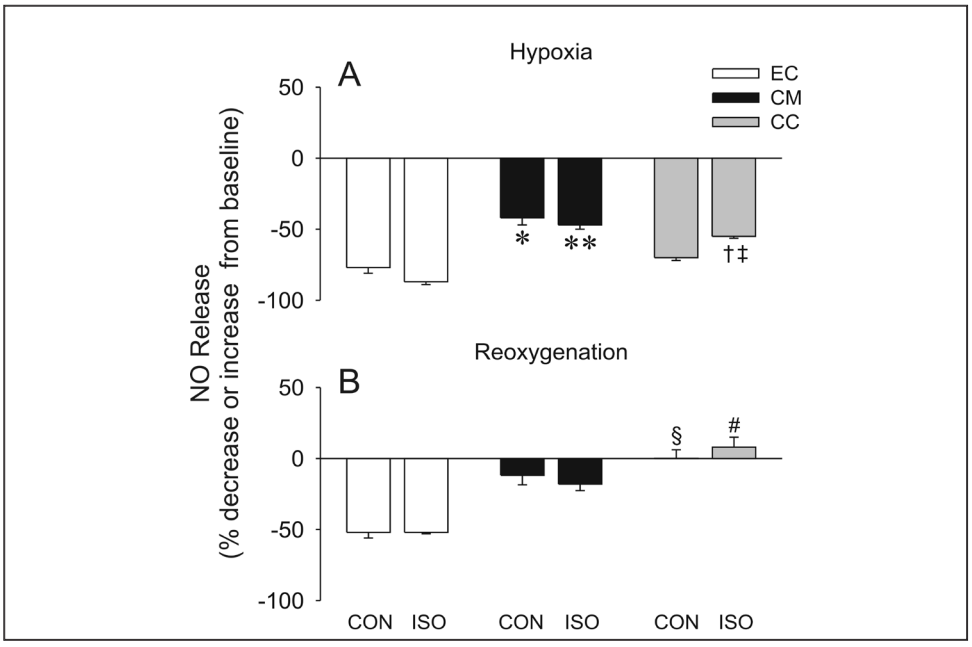

Fig. 5. Histograms depicting nitric oxide (NO) production in endothelial cells (EC), cardiomyocytes (CM) and co-cultures (EC and CM cultured together; CC) in the presence (ISO) or absence (CON) of isoflurane during hypoxia (panel A) and reoxygenation (panel B).

*NO-levels in pmol per mg protein, expressed as % decrease or increase from baseline. Data are mean ± SEM (n=6/group); *P < 0.05 vs. CON EC (hypoxia), **P < 0.05 vs. ISO EC (hypoxia), †P < 0.05 vs. CON co-culture (hypoxia), ‡P < 0.05 vs. ISO EC (hypoxia), §P < 0.05 vs. CON EC (reoxygenation), #P < 0.05 vs. ISO EC (reoxygenation).*

3.4. *mPTP opening is delayed in EC–CM co-culture*

Protection of mitochondria during reperfusion is likely to be a critical component involved in the salvage of myocardial tissue induced by ischemic- and pharmacological pre- or post-conditioning. A key component in the process appears to be modulation of the mPTP. Opening of the mPTP results in collapse of the mitochondrial membrane potential and subsequent disruption of normal mitochondrial function. CM cultured in the presence of EC demonstrated a delay in mPTP opening when compared to CM alone, and this delay was augmented by isoflurane treatment (Fig. 6).

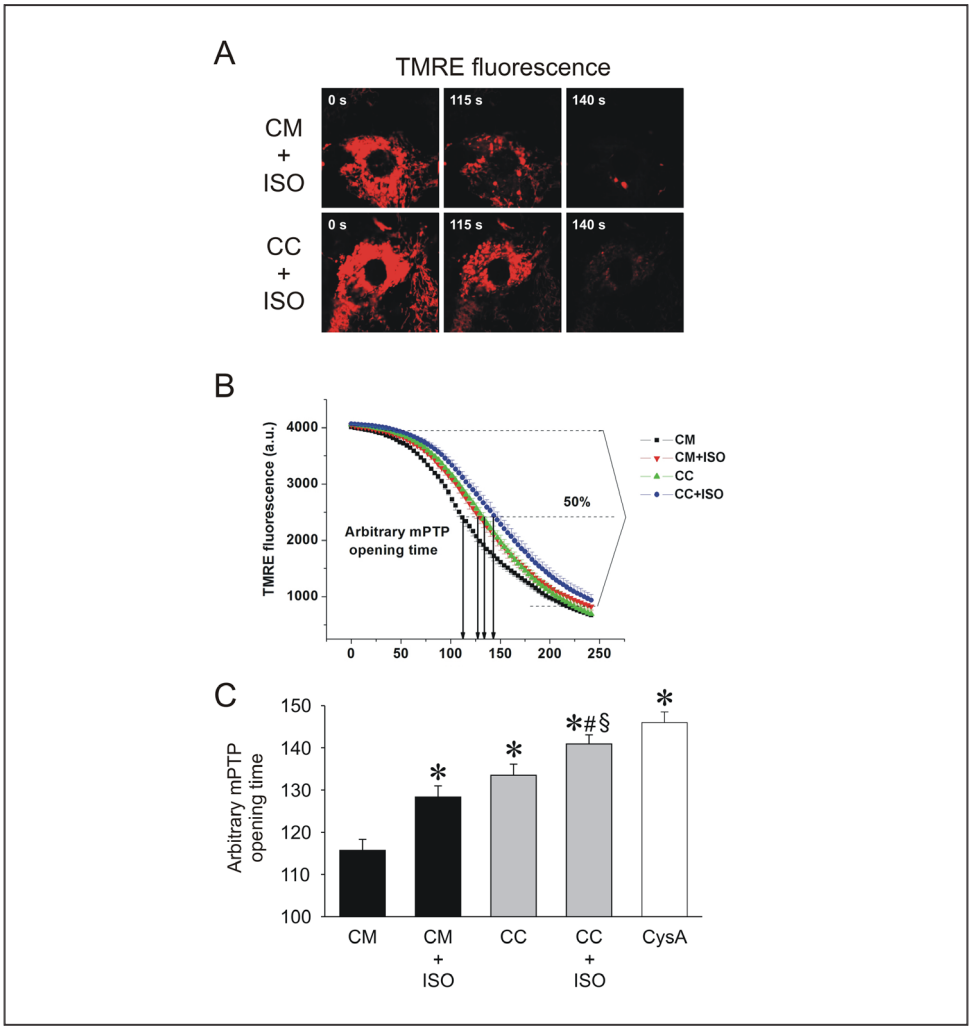

Fig. 6. (A) Photoexcitation-generated oxidative stress induces mitochondrial permeability transition pore (mPTP) opening as observed by the rapid dissipation of tetramethylrhodamine ethyl ester (TMRE) fluorescence in isoflurane (ISO) stimulated cardiomyocytes (CM+ISO) and co-culture (EC and CM cultured together; CC+ISO). (B) The time by which TMRE fluorescence intensity decreased by half between initial and residual fluorescence intensity was considered as the „arbitrary mPTP opening time" (arrows) and was compared among experimental groups (a.u. = arbitrary units). (C) shows a histogram representing the arbitrary mPTP opening times.

*CysA = cyclosporine A (n=8/group). Data are mean ± SEM; *P < 0.05 vs. CM, #P < 0.05 vs. CM+ ISO, §P < 0.05 vs. CC.*

3.5. *HIF1α suppression in EC decreases eNOS expression and phosphorylation, and abolishes the protective effects of isoflurane in EC–CM co-culture*

EC infected with lentiviral vector expressing short hairpin shRNA over a 72 h period demonstrated an 80–90% decrease in HIF1α expression (Fig. 7A). Silencing of HIF1α in EC abolished isoflurane-induced protection of CM against H/R (Fig. 7B) in co-culture. In contrast, EC infected with a lentiviral vector expressing a scrambled sequence had no effect on isoflurane-induced reduction in LDH release. eNOS expression in EC treated with lentivirus containing shRNA for HIF1α was significantly reduced. In addition phosphorylation of eNOS was reduced in L-NAME treated EC and not detectable in shRNA treated EC (Fig. 7C). CM failed to express eNOS at any timepoint during the experiment (Supplemental data Fig. 3). These results are consistent with our findings that basal and isoflurane-stimulated NO production in EC treated with HIF1α shRNA was significantly decreased (Table 1). Taken together, the data support the hypothesis that isoflurane mediated protection of CM is dependent upon endothelial expression of HIF1α, HIF1α-dependent expression of eNOS, and production of bioavailable NO.

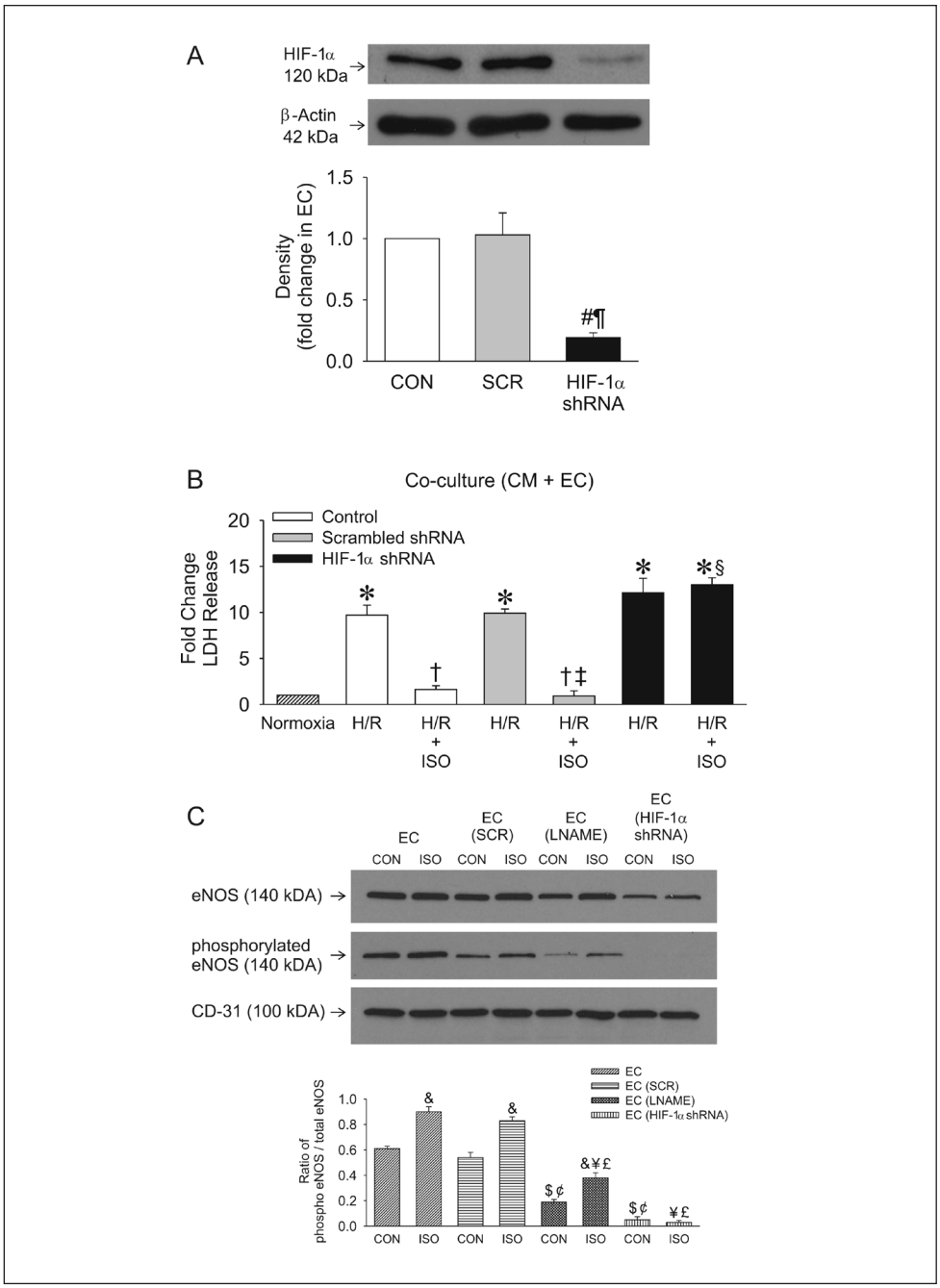

Fig. 7. Panel (A) representative Western blot depicting HIF1α protein in uninfected EC (CON), EC with lentiviral vector carrying a scrambled sequence (SCR) and EC infected with lentivirus containing shRNA for HIF1α.

HIF1α immunoreactive bands are expressed as fold change over CON (n=6/group).

Panel (B) summarizes lactate dehydrogenase (LDH) activity in co-cultures (CM+EC) with uninfected endothelial cells (EC, control), EC infected with lentivirus expressing scrambled shRNA (scrambled shRNA) and lentiviral infection of EC with shRNA for hypoxia-inducible factor (HIF1α shRNA).
Co-cultures were subjected to hypoxia and reoxygenation (H/R) in the presence or absence of isoflurane (ISO). LDH activity is expressed as fold change over the normoxic-group (n=6/group).

Panel (C) representative Western blots showing eNOS and phosphorylated eNOS expression in uninfected EC, EC with lentiviral vector carrying a scrambled sequence (SCR), EC in the presence of NG-nitro-L-arginine methyl ester (L-NAME) and EC infected with lentivirus containing shRNA for HIF1α (shRNA), subjected to H/R in the presence or absence of ISO (n=6/group).
*Data are mean ± SEM; *P < 0.05 vs. normoxia, †P < 0.05 vs. H/R (control), ‡P < 0.05 vs. H/R (scrambled shRNA), §P < 0.05 vs. H/R+ISO (scrambled shRNA), #P < 0.05 vs. CON, ¶P < 0.05 vs. SCR, &P < 0.05 vs. respective control (CON), $P < 0.05 vs. EC CON, ¢P < 0.05 vs. EC (SCR) CON, ¥P < 0.05 vs. EC ISO, £P < 0.05 vs. EC (SCR) ISO.*

4. Discussion

eNOS-derived NO has been identified as a critical trigger of cardioprotection that is recruited by endogenous pathways [28] and by the volatile anesthetic isoflurane [19]. Studies investigating protection against myocardial ischemia and reperfusion injury have focused primarily on NO derived from a NOS isoform in CM, but our data indicate that EC are an underappreciated and important paracrine source of NO during cardioprotection. Cardiac endothelial cell–myocardial signaling has been described in the context of heart development, modulation of contractility, control of rhythmicity, and in the pathogenesis of heart failure [1], however, little is known regarding the specific contribution of EC to CM protection against ischemia and reperfusion injury. Cardiac EC outnumber CM *in vivo* by 3:1, although, the mass ratio of these two cell types is .04–.05 [29]. This abundance of EC in the heart provides a diffusion radius between EC and CM sufficient for effective EC–CM-NO signaling even considering the short biological half life of NO. Thus, EC–CM interactions are likely to play a critical role that influences outcome during pharmacological protection against myocardial injury, and the current results support the concept that this action occurs through distinct signaling events in EC versus CM.

The transcription factor HIF1α has been demonstrated to play an important role during hypoxic- and ischemic-preconditioning in the myocardium and brain [30,31], and is involved in anesthetic-induced organ protection [21,22].

HIF1α is a heterodimeric protein composed of a constitutively expressed α-subunit and an α-subunit under regulatory control. Functional control of the concentration of HIF1α in tissue occurs by changes in both expression and post-translational stabilization of the protein [32]. Hypoxia is the predominant stimulus for production of HIF1α; however non-hypoxic stimuli including reactive oxygen species (ROS), insulin, thrombin, growth factors, and cytokines can modify HIF1α expression [33–35]. Primary regulation of HIF1α under normoxic conditions occurs via specific hydroxylation and subsequent degradation by prolyl hydroxylase (PHD) within the oxygen-dependent domain [36]. Although the activity of PHD is oxygen-dependent, this enzyme is also regulated by ROS and NO [37]. Thus, deactivation of PHD by NO could suggest the presence of a positive feedback loop between NO and HIF1α activation. Hydroxylation prevents transactivation of HIF1α to the nucleus under normal oxygen tension. However, during hypoxia HIF1α is not subjected to proteasomal degradation, but rather, is translocated to the nucleus where it induces expression of proteins (e.g. VEGF, erythropoietin, hemoxygenase and GLUT1 transporter) associated with protection against hypoxic injury [38,39]. Increases in the expression of HIF1α have been observed in cells under normal oxygen tension, suggesting that alternate signaling pathways may also regulate the expression of this important transcription factor.

The current results confirm and extend previous findings by demonstrating that isoflurane enhanced CM survival in the presence of EC by differentially regulating HIF1α and modulating NO production in EC versus CM. Pharmacological stimulation with isoflurane produced an increase in cytosolic and nuclear expression of HIF1α in EC, but not in CM, and this action was blocked by inhibition of ERK with either of two distinct MEK inhibitors. Isoflurane has previously been shown to increase release of signaling ROS by mitochondria [10] which could result in ERK activation via redox regulation of mitogen activated protein kinase phosphatases [40]. Although we previously observed increased HIF1α expression in the myocardium, isoflurane did not increase HIF1α in isolated CM in the present investigation [21]. This finding might be explained by a lack of discrimination between proteins expressed in specific cell types in the whole heart using Western blotting previously, or because of species-dependent (i.e. rat versus rabbit) differences in the magnitude or time-dependence of cell signaling events. For example, isoflurane stimulated the expression of HIF1α in Hep3B cells in a time-dependent manner [22]. However, maximal expression of HIF1α occurred between 4 and 8 h after exposure to iso-

flurane in Hep3B cells, whereas, our results in EC indicate a more rapid (1 h) increase in expression. During co-culture of EC and CM, isoflurane produced significant protection of CM against hypoxia and reoxygenation injury, but, this beneficial effect was abolished by suppressing HIF1α expression in EC with lentiviral shRNA. Thus, the findings support the contention that signals initiated in the endothelium by pharmacological agents such as volatile anesthetics result in the transference of factors that mediate protection of CM in a paracrine fashion.

VEGF is a recognized downstream target regulated by HIF1α [41,42] and our previous findings demonstrated that isoflurane administered prior to coronary artery occlusion in rats resulted in enhanced myocardial expression of HIF1α and VEGF [21]. HIF1α expression appeared to be essential for induction of VEGF mRNA and expression of VEGF protein, actions that were inhibited by HIF1α knockdown with siRNA in basophils [43]. VEGF is also known to stimulate an increase in NO production [44], however, it is unknown if increases in VEGF in EC were specifically responsible for HIF1α-mediated increases in NO production and subsequent protection of CM against injury observed during isoflurane in the present study. This possibility is currently under investigation.

The non-selective NOS inhibitor L-NAME abolished the protective effect of isoflurane on CM in co-culture when administered either throughout experimentation or only during reoxygenation. In contrast, the presence of L-NAME only before hypoxia had no effect on CM protection. These results suggest that NO is an important mediator of pharmacological protection by isoflurane, consistent with previous findings in $eNOS^{-/-}$ mice [19]. Furthermore, silencing of HIF1α resulted in a significant decrease in EC-derived eNOS and phosphorylated eNOS that likely explains the dramatic reduction in NO production. CM failed to express eNOS protein at any timepoint during the experiment supporting the concept of endothelial derived NO in our co-culture model. These data suggest that expression of HIF1α during normoxic conditions is important for sustained expression and phosphorylation of eNOS and thus production of NO. Importantly, HIF1α may also be important for maintaining eNOS expression during hypoxia and reoxygenation as silencing of HIF1α resulted in a further decrease in eNOS expression under these conditions.

The targets of EC-derived NO within CM that are related to cytoprotection have been incompletely elucidated, although, evidence suggests that NO may have direct effects on specific mitochondrial proteins. For example,

direct or indirect effects of NO on the mPTP have been implicated in mechanisms responsible for cardioprotection [45]. Pharmacological protection against ischemia and reperfusion injury with isoflurane has been shown to be dependent on inhibition of this somewhat elusive protein complex [19,25]. The present results confirm earlier findings and further demonstrate that the presence of EC delays opening of the mPTP in CM subjected to laser induced injury. Remarkably, these beneficial actions were augmented by isoflurane. NO released by EC may produce favorable effects on CM by binding to heme-containing proteins such as cytochrome c oxidase: an effect that inhibits its activity. This action would be expected to modulate terminal electron transfer to molecular oxygen resulting in the attenuation of cellular respiration and potentially decreasing the formation of damaging ROS [46] that promotes mPTP opening.

Notably, the current findings support the existence of crosstalk between EC and CM that contributes to cardioprotective signaling, and indicate that NO is an important mediator during cell–cell communication. Isoflurane stimulated an increase in NO production from EC under baseline conditions, but, did not significantly increase NO production in co-culture. The lack of increase of NO observed under these conditions may have been related to fewer total EC in co-culture (1:12 EC–CM) as compared with EC cultured alone. Alternatively, myoglobin is capable of binding NO to form iron-nitrosyl myoglobin and CM have been shown to scavenge free NO [47]. Hypoxia profoundly decreased NO production in EC, CM and in EC–CM co-culture, however, NO concentrations were nearly two fold higher during hypoxia in isoflurane-treated compared to untreated-cells in co-culture. This finding suggests that volatile anesthetics may preserve the mechanisms that are responsible for regulating NO production by NOS [17] or by influencing nitrite/nitrate metabolism during hypoxia. Interestingly, NO concentrations remained depressed after reoxygenation in EC or CM alone, but, were restored to baseline values in co-culture. This observation supports the idea that CM may reciprocally influence EC by secreting additional paracrine factors that impact NO production by EC. For example, the alarmin cytokine High Mobility Group Box 1 has been shown to be released by necrotic or injured CM [48] and this protein is a ligand for the EC receptor for advanced glycosylation end-products (RAGE) [49]. The latter has been shown to be upregulated during hypoxia and also negatively regulates NO production [50]. The role of alarmins or of other candidate proteins (e.g. neuregulin, PDGF-B or angiopoietin-1) [51,52] to modulate EC–CM crosstalk and influence CM survival after hypoxia and

reoxygenation remains to be elucidated. Although isoflurane did not further improve NO production by EC and CM after hypoxia and reoxygenation, isoflurane did enhance CM survival in co-culture. This finding may suggest that NO production during hypoxia or the initial period of reoxygenation is the most critical in eliciting isoflurane-induced cardioprotection.

The results of the current investigation support the contention that paracrine and autorcrine factors contribute to protection of CM against hypoxia and reoxygenation injury. However, interpretation of the data is subject to certain limitations. We used neonatal CM rather than adult CM because neonatal CM can be readily cultured over several days and retain a beating phenotype. Although the expression of neonatal isoforms of certain proteins may not fully recapitulate the expression profile in adult CM, the protection of neonatal CM by isoflurane is consistent with that found in the adult heart. In addition, adult CM lose their ability to beat spontaneously when in culture and undergo morphological (decrease in cell size), electrophysiological (changes in cardiac ion channel profile and cell capacitance) and structural changes (myofibrillar damage, substantial decrease in T-tubule density), that might alter cellular signaling events [53,54].

In conclusion, the results demonstrate that pharmacological induction of HIF1α in EC by a volatile anesthetic agent promotes cardioprotection of CM against hypoxia and reoxygenation injury, and this action is NO-dependent and associated with inhibition of mPTP opening in CM. The results further suggest that crosstalk between EC and CM contributes to cardioprotection.

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Disclosures

None.

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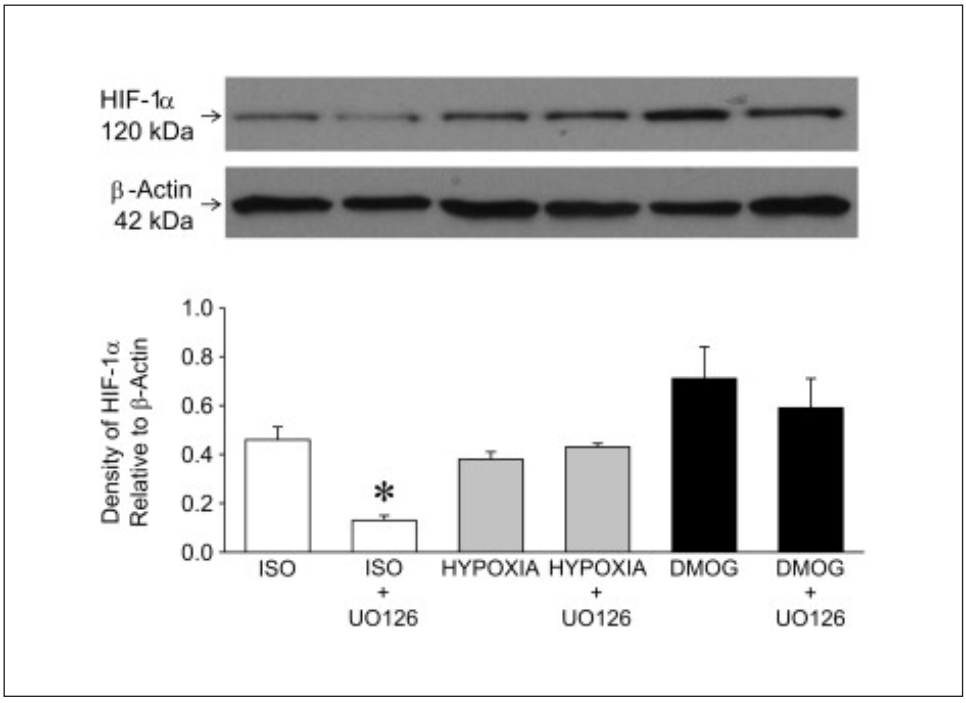
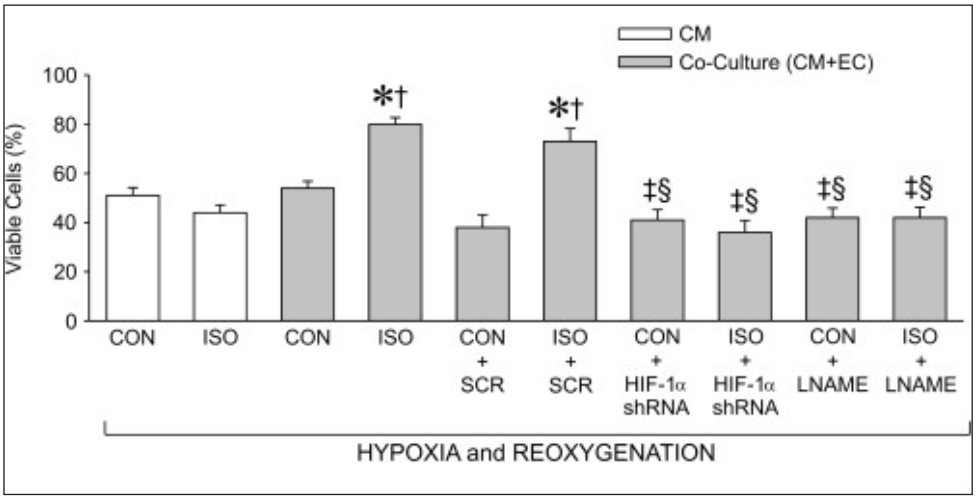
Supplemental data



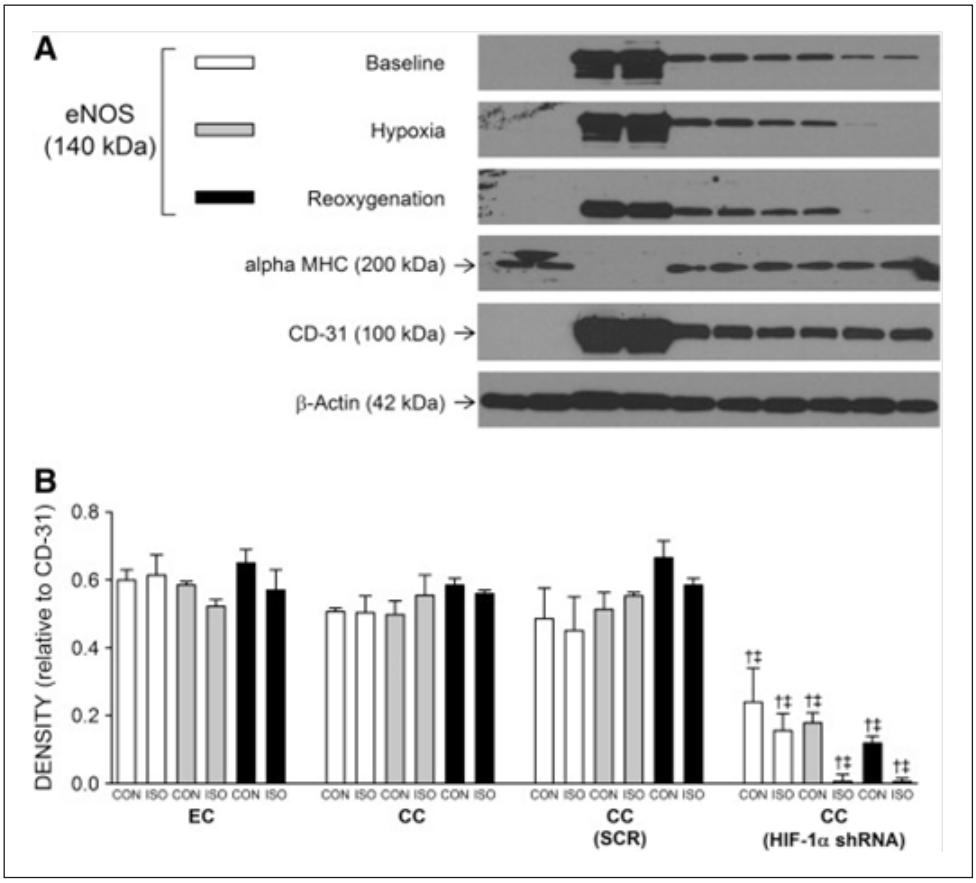
Supplement Fig. 1. Representative Western blots showing isoflurane (ISO; 60 min), hypoxia (120 min) and dimethyloxaloylglycine (DMOG; 120 min) treated endothelial cells (EC) in the presence or absence of Mitogen-activated Protein/Extracellular Signal-regulated Kinase inhibitor (MEK) U0126. All three stimuli were able to upregulate hypoxia inducible factor 1α (HIF1α) protein expression in EC. However, UO126 treatment only abolished isoflurane induced HIF1α expression and had no significant effect on hypoxic or DMOG treated EC. These results suggest that MEK, an upstream kinase that phosphorylates extracellular signal-regulated kinase, is crucial for isoflurane mediated pathways resulting in upregulation of HIF1α. HIF1α protein amount normalized to β-Actin.

*Data are mean ± SEM (n = 4/group); *P < 0.05 vs. ISO.*



Supplement Fig. 2. Trypan blue exclusion assay expressing percentage of viable cells to total cells counted. These results are consistent with our data using lactate dehydrogenase (LDH) as an index of cell injury. The protective effect of isoflurane was abolished by silencing of hypoxia inducible factor 1 (HIF1α) in EC or N^G-nitro-l-arginine methyl ester (l-NAME) treatment. Trypan blue exclusion experiments in endothelial cells (EC) cultured alone showed no decrease of viability when subjected to hypoxia and reoxygenation (data not shown).

*Data are mean ± SEM (n = 6/group); *P < 0.05 vs. respective control (CON); †P < 0.05 vs. cardiomyocytes (CM) ISO;‡P < 0.05 vs. co-culture ISO; §P < 0.05 vs. co-culture SCR +*



Supplement Fig. 3. (A) Representative Western blots showing eNOS protein expression in cardiomyocytes (CM), endothelial cells (EC) and co-culture (CC) in the presence (ISO) or absence (CON) of isoflurane at baseline, hypoxia and reoxygenation. Western blots are overexposed (30 min) to maximize the detection of eNOS expression in shRNA treated CC, however, shorter exposure times were used for densitometric analysis. eNOS was not detected in CM at any timepoint in the experiment. Panel (B) data are normalized to the EC-marker CD-31, because CM failed to express eNOS protein. shRNA treatment of EC (3 day protocol) before co-culture significantly decreased eNOS expression at baseline, hypoxia and reoxygenation. No significant changes in eNOS protein expression were detected in EC, CC and CC (SCR). Protein samples were probed for alpha-myosin heavy chain (CM-marker), CD-31 (EC-marker) and β-actin to demonstrate the presence of both cell types in our co-culture (representative blots are shown in panel A). Phosphorylated eNOS was not detected in the co-culture groups, which may be attributed to the small number of EC in our co-culture (1:12).

Data are mean ± SEM (n = 4/group); †P < 0.05 vs. respective sample CC; ‡P < 0.05 vs. respective sample CC (SCR).